Diagnosing Flu

A new Livermore tool can quickly tell

PEOPLE with flulike symptoms who seek treatment at a medical clinic or hospital often must wait several hours before being examined, possibly exposing many people to an infectious virus. If a patient appears to need more than the routine fluids-and-rest prescription, effective diagnosis requires tests that must be sent to a laboratory. Hours or days may pass before results are available to the doctor, who in the meantime must make an educated guess about the patient's illness. The lengthy diagnostic process places a heavy burden on medical laboratories and can result in improper use of antibiotics or a costly hospital stay.

A faster testing method may soon be available. An assay developed by a team of Livermore scientists can diagnose influenza and other respiratory viruses in about two hours once a sample has been taken. Unlike other systems that operate this quickly, the new device, called $FluID_x$ (and pronounced "fluidics"), can differentiate five types of respiratory viruses, including influenza. $FluID_x$ can analyze samples at the point of patient care—in hospital emergency departments and clinics—allowing medical providers to quickly determine how best to treat a patient, saving time and potentially thousands of dollars per patient.

The $FluID_x$ project, which is led by Livermore chemist Mary McBride of the Physics and Advanced Technologies Directorate, received funding from the National Institute of Allergy and Infectious Diseases and the Laboratory Directed Research and Development (LDRD) Program. To test the system and make it as useful as possible, the team worked closely with the Emergency Department staff at the University of California (UC) at Davis Medical Center in Sacramento. Robert Derlet, M.D. and chief of the department, is enthusiastic about having $FluID_x$ available for testing.

"A dozen or more viruses cause symptoms in people that all look the same in the early stages," says Derlet. "With most viruses, people are sick for just a few days and then get better. But flu and other

In the background, a micrograph shows the morphologic features found in the 1918 influenza virus. (Photographer: Cynthia Goldsmith. Reprinted courtesy of the Centers for Disease Control and Prevention.)

Fast

which patients have influenza or another respiratory virus.

respiratory viruses can make some people really sick and even kill them. We need to be able to sort out the 'bad guys' so these viruses don't infect others."

From the Lab to the Clinic

Flu kills more than 35,000 people every year in the U.S. (See the box on p. 7.) The 2003 outbreak of severe acute respiratory syndrome and the ongoing concern about a possible bird flu pandemic illustrate the need for a fast, reliable test that can differentiate seasonal flu from a potentially pandemic influenza. Such a test should also discriminate influenza from pathogens that cause illnesses with flulike symptoms.

When a precise diagnosis is required to treat an adult patient with serious respiratory symptoms, sample cells are usually obtained with a nasal or throat swab and analyzed with one of several laboratory methods. The gold standard test is viral culturing, a highly sensitive method that can identify the specific strain of virus. However, viral culturing is a labor-intensive process and requires 3 to 10 days to produce results, far too long for early intervention. Enzyme and optical immunoassays offer results in 30 minutes, but these methods are less sensitive than viral culturing so they can produce false positives or negatives. They also cannot distinguish the type of virus found.

Direct immunofluorescence antibody (DFA) staining is as sensitive as viral culturing. It also can detect multiple respiratory pathogens simultaneously by a process known as multiplexing. However, DFA staining requires expensive equipment, a skilled microscopist, and samples with enough target cells for testing. In addition, the results are ultimately subjective. Another method, called reverse transcriptase-polymerase chain reaction assay, offers sensitivity and specificity comparable to viral culturing and DFA staining. It also produces results in two hours and can rapidly test a large number of samples. The drawback with these tests, however, is that they must be performed in a laboratory. None of them can be used where they are needed most: in the clinic or emergency department where patients are being treated.

FluID$_x$ team members (from left) Jim Birch, Jack Regan, and Kristl Adams view results from a recent test. The system can diagnose influenza and other respiratory viruses in about two hours after a sample is taken.

Lawrence Livermore National Laboratory

FluID$_x$ team member Sonia Letant works on assay development.

Livermore's FluID$_x$ diagnostic system, with its instrumentation and multiplexed assays, is designed specifically for point-of-care diagnosis. The fast, easy-to-use system is based on the Autonomous Pathogen Detection System, a homeland security technology developed by Lawrence Livermore. This R&D 100 Award–winning technology constantly monitors the air to detect airborne bioterrorism agents, such as anthrax. (See *S&TR*, October 2004, pp. 4–5.)

FluID$_x$ is an integrated system designed to perform highly multiplexed polymerase chain reaction (PCR) nucleic-acid-based assays in real time. The FluID$_x$ system processes a sample, analyzes the data, reports the results, and decontaminates itself before another sample is taken. The device currently uses 16 assays—12 for individual nucleic-acid targets and 4 for internal controls. The assays can simultaneously detect influenza A and B, parainfluenza (Types 1 and 3), respiratory syncytial virus, and adenovirus (Groups B, C, and E).

Process for Assay Development

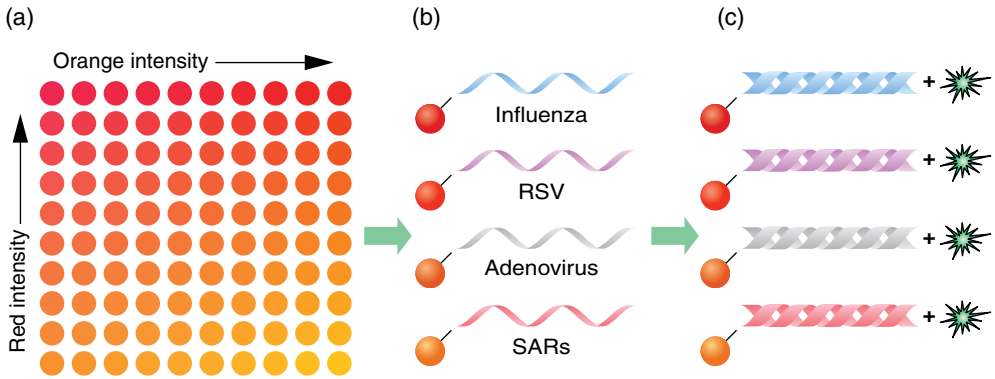
FluID$_x$ works so well because its nucleic-acid-based detection assays have been carefully vetted using a proven assay-development process. As a result, the assays produced are extraordinarily sensitive, specific, and robust.

The process begins with an analysis of all available genomic sequence information, which forms the basis for developing a signature—the region or set of regions on a chromosome that is unique to the target organism. Traditional approaches to developing DNA signatures start by identifying a gene that is considered vital to the target organism's virulence, host range, or other distinctive factor.

Livermore's bioinformatics team has developed a whole-genome comparative analysis software system called KPATH to improve the signature-mining process. (See *S&TR*, April 2004, pp. 4–9.) KPATH aligns multiple genomes of different strains of the target species and compares them to identify all the genomes that contain the target sequence. These genomes are then compared with Livermore's immense database of microbial organisms to establish that the organism-conserved target sequence does not occur in other sequenced microbial organisms. An algorithm subtracts any target sequence already listed in the microbial organism database. The resulting sequence is mined for potential signature candidates. Additional electronic screening catches nonexact matches that might produce false positives.

The signature-mining process then moves from the computer to the laboratory to further reduce the field of candidates. Potential nucleic-acid signatures are screened against more than 2,500 randomly selected nucleic-acid extracts from soils, microbes, bacteria, and other organisms that may be present in a sample when it is collected. Candidate signatures are also exposed to near-neighbor nucleic acids to reduce the potential for false results.

Signatures that pass the intensive background screening in real-time PCR are developed into assays in a format



(a) The FluID$_x$ system uses a liquid array of 100 beads, each with a unique spectral value. (b) Nucleic-acid probes capture beads that complement the target pathogens: influenza, respiratory syncytial virus (RSV), adenovirus, and severe acute respiratory syndrome (SARS). (c) Amplified nucleic acid is hybridized to fluorescently labeled beads and analyzed.

known as Taqman. At this stage, every parameter that might affect an assay's performance is optimized, and all assays are fully characterized against a standardized panel of targets and near-neighbors. The final results are captured in a certificate of analysis, providing a record of the assay's pedigree.

For the multiplexed liquid array format, individual signatures are added to the multiplexed PCR mix, with other panel signatures present. Signatures are tested several times to determine an assay's detection limit for each target and to control for reactivity among signatures.

FluID$_x$ at Work

The multiplexed nucleic-acid assays in FluID$_x$ use tiny polystyrene microbeads colored with unique ratios of red- and orange-emitting dyes. The result is an array of 100 beads that can be distinguished by their spectral values. The beads are coated with nucleic-acid probes whose sequences complement those of the five target pathogens. Flow-through PCR amplifies nucleic acids from the viruses, and the amplified DNA, or amplicons, are introduced to the beads. The amplicons hybridize to their complementary probes on the appropriate bead. Then the beads are illuminated with red laser light, which has a wavelength of 635 nanometers, to classify each bead. Next, illumination with 532-nanometer (green) laser light quantifies the assay at the bead's surface based on the strength of the fluorescence. Conducting the multiplexed assay requires several steps and significant PCR thermocycling times, resulting in the two-hour duration for a FluID$_x$ test.

Four control beads in every sample convey information about the system's status. For example, the instrument control bead verifies the optics of the green laser. This bead is coated with a probe that is unlikely to bind to other nucleic acids and with a dye that emits a constant fluorescence in all samples. A change in fluorescence on the control bead indicates a fluctuation in the green laser's performance. FluID$_x$ analyzes every sample in the context of all four controls, thereby minimizing the likelihood of instrument malfunction or false results.

Livermore was not the first institution to use coated microbeads as indicators. However, says McBride, "We have put them to excellent use, applying them in several biosensors." In the FluID$_x$ system, the beads' fluorescence signal above or below a threshold value indicates whether the assay is ruled positive or negative.

The FluID$_x$ sample preparation module and its detector are composed of commercially available parts with customized interfaces. The automated flow-through PCR unit, which was designed at Livermore, consists of a custom thermocycler with a copper heater mounted in line with the sample preparation unit. (See the figure on p. 8.) The software used in FluID$_x$ is similar to that developed for the Autonomous Pathogen Detection System, but its user-interface has been designed so that personnel can operate the system with minimal training. This version is easy to use, readily accommodates hardware changes, and enables communication with

What Is Flu?

Seasonal influenza, or the flu, is a contagious respiratory illness caused by viruses that spread in droplets from coughing and sneezing. Most adults may be able to infect others beginning one day before symptoms develop and up to five days after they become sick. Symptoms can include fever, headache, fatigue, cough, sore throat, runny nose, or muscle ache. Flu may cause only mild illness but also may lead to severe illness or even death.

Although seasonal flu is often thought of as a trivial illness, statistics tell a different story. Every year in the U.S., 5 to 20 percent of the population typically gets the flu. More than 200,000 people are hospitalized from flu complications, and about 36,000 people die from flu. Fortunately, most people have some immunity to seasonal influenza, and a vaccine is developed annually.

Common flu is usually caused by an influenza A virus. Avian, or bird, flu is a subtype of the influenza A virus that occurs naturally among birds. Different subtypes are caused by changes in certain proteins, such as hemagglutinin (HA) and neuraminidase (NA), on the surface of the influenza A virus and by the way the proteins combine. Some subtypes cause only mild symptoms. The bird flu subtype A/H5N1 is highly pathogenic, spreading rapidly among flocks of poultry. Its mortality rate can reach 90 to 100 percent, often within 48 hours.

Among humans, only three known influenza A subtypes are in circulation: A/H1N1, A/H1N2, and A/H3N2. The A/H5N1 bird flu variant is the most deadly of the few avian viruses to have crossed the species barrier to infect humans. Humans have no immunity to A/H5N1, and no vaccine is currently available. Since 2003, human A/H5N1 cases have been reported in Azerbaijan, Cambodia, China, Dijbouti, Egypt, Indonesia, Iraq, Thailand, Turkey, and Vietnam. More than half of the people infected with the A/H5N1 virus have died. To date, no human-to-human transmission of the disease has been sustained, but influenza viruses mutate rapidly. Health officials are concerned that A/H5N1 will evolve into a virus that can be transmitted from person to person.

Confusingly for physicians, other influenzalike illnesses start out looking almost exactly the same as common flu. Adenoviruses can affect not only the respiratory system but also the eyes, intestines, and urinary tract, most commonly in children. Parainfluenza and respiratory syncytial virus are common causes of lower respiratory tract disease in young children, and both can cause repeated infections throughout life. A tool such as FluID$_x$ will help physicians quickly determine the cause of a patient's flulike symptoms.

outside entities via an Ethernet connection or other medium.

Put to the Test

In tests of FluID$_x$, a technician at the UC Davis Medical Center collected more than 1,200 nasal swab samples from patients seeking treatment in the Emergency Department. As part of the federal requirements for research involving human subjects, study participants signed informed consent forms before samples were taken, and the testing protocol was approved by UC Davis physicians and the governing Institutional Review Board.

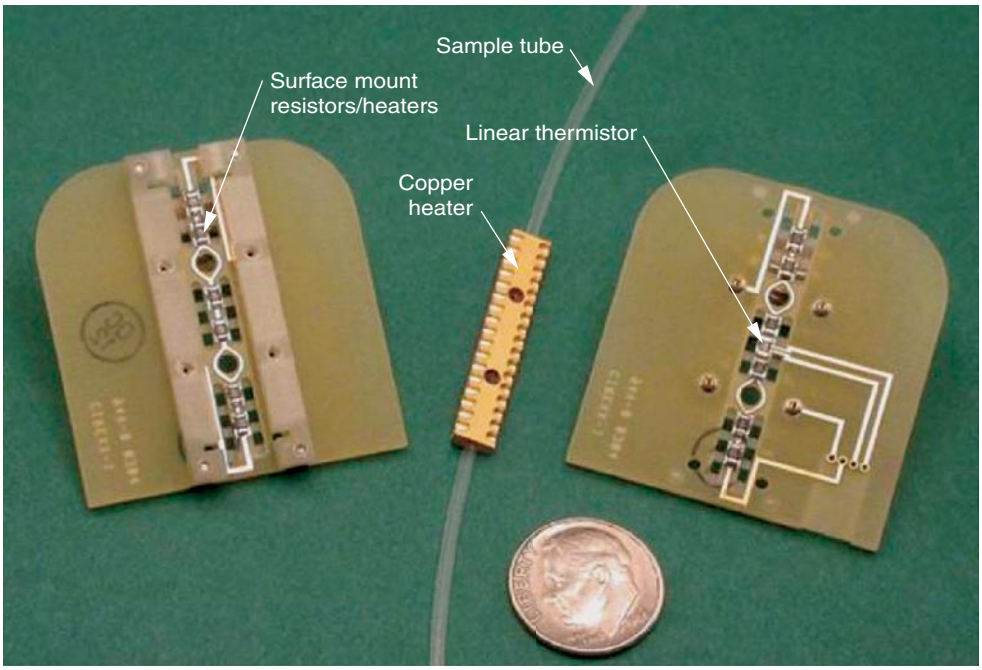
Nasal swabs were also collected from volunteers who showed no signs of illness. All samples were processed using viral culturing, DFA staining, and FluID$_x$. Sample comparisons revealed excellent results for FluID$_x$. In terms of sensitivity, the FluID$_x$ multiplexed assays were on par with the results from viral culturing. The system's specificity for identifying virus strains was significantly better than that obtained with DFA staining, which takes about the same time as FluID$_x$.

The Work Goes On

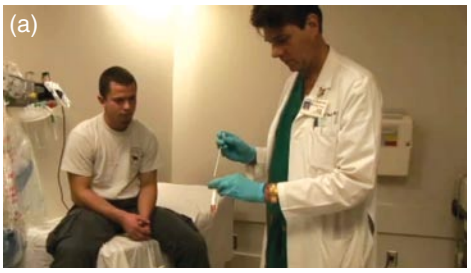
The Laboratory works closely with the Centers for Disease Control and Prevention (CDC), which provided a few assays for the FluID$_x$ panel. Livermore is part of the CDC Laboratory Response Network that would be activated in the event of a widespread flu pandemic. If the FluID$_x$ system can be made small enough to be taken into the field, it could be used by emergency responders. A field-portable unit would be invaluable if a flu pandemic were to occur.

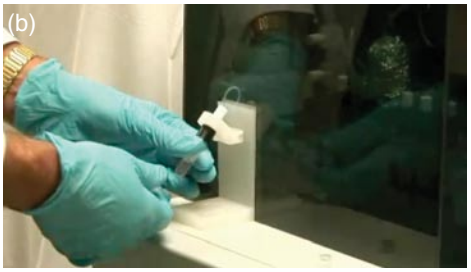
McBride and her team are developing additional assays that could be used to detect avian influenza (bird flu). Although the current panel can detect influenza A and B, it cannot differentiate normal seasonal flu from a potentially pandemic influenza. Influenza A is divided into subtypes based on the properties of two surface antigens, hemagglutinin (HA) and neuraminidase (NA). The HA antigen has 15 subtypes, and the NA has 9. Bird flu is subtype H5N1, while seasonal influenza is usually subtypes H1, H2, or H3.

The team used KPATH to identify and select most of the candidate signatures included in the FluID$_x$ respiratory panel. But KPATH could not identify nucleic-acid signatures in the required format for the HA gene, which is the basis for identifying the H subtypes of influenza A. Assays developed to operate in the Taqman PCR



Livermore's automated flow-through polymerase chain reaction thermocycler includes a circuit-board heater and a copper heater.





The FluID$_x$ device was tested in the Emergency Department of the University of California at Davis Medical Center. (a) Robert Derlet (right), M.D. and department chief, inserts a nasal swab sample collected from a volunteer into a vial and (b) places the vial in the FluID$_x$ device. (c) Less than two hours later, a screen read-out provides test results.

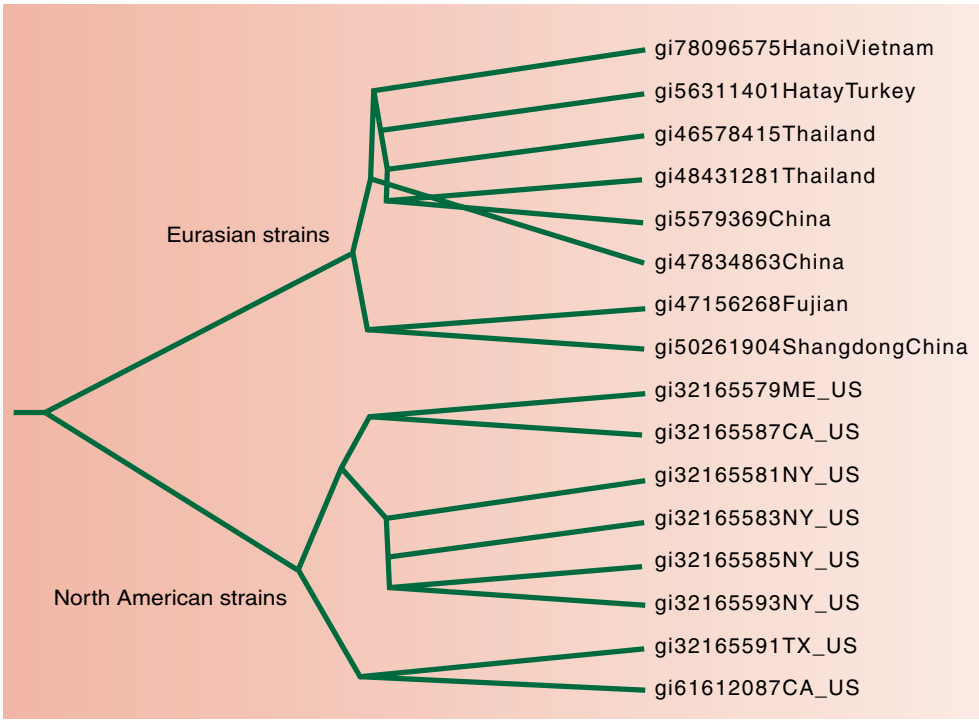
format handle one signature at a time. The HA gene, however, is highly divergent, so no single Taqman signature can capture all members of a subtype.

To identify the smallest number of signatures required to recognize all members of a target set, the team used the Livermore software Minimal Set Clustering. This software generates sets of Taqman signatures that it predicts can be used in combination to detect all of the genome target sequences. Both KPATH and Minimal Set Clustering ensure that the signatures are predicted not to cross-react with a sequenced nontarget organism.

In designing signatures for the influenza A subtype called H5, McBride's team obtained a target set of 217 H5 sequences from Genbank, which is part of the National Center for Biotechnology Information. Minimal Set Clustering showed that four Taqman signatures are needed to detect all members of this target set. The H5 subgroups also appear to cluster by lineage, indicating that the signatures may discriminate where a strain originated. The first and fourth signatures detect groups of North American sequences, and the second and third detect Eurasian sequences. This distinction is critical because, to date, all avian flu strains infecting humans have been of Eurasian lineage. Similar work revealed the minimal number of signatures needed to provide complete coverage for the other influenza A subtypes, H1, H2, H3, and H7.

McBride and her team are also working with the CDC's Bioterrorism Rapid Response and Advanced Technology Laboratory, which will have lead responsibility for analyzing samples during a bioterror attack. Although the FluID$_x$ instrument is designed for use in hospitals and medical clinics, the technology can be adapted for any situation in which rapid biological assays are needed.

In October, the team submitted the FluID$_x$ system to the U.S. Food and Drug Administration to have it approved as a medical device. "Approval will likely take about a year," notes McBride. The Laboratory is already meeting with potential industrial partners who are interested in licensing the FluID$_x$ technology for commercial use.



Minimal Set Clustering software identifies the fewest number of signatures required to detect all members of a divergent target set. The branches on this phylogenetic tree show the four signatures needed to diagnose the relevant HA5 sequences of influenza A. The top two signatures target Eurasian strains, and the bottom two target North American strains.

In the meantime, the team continues to improve the assays and is working to automate a stand-alone device. The LDRD Program is supporting assay development to expand the FluID$_x$ panel. Device testing will continue at the Emergency Department of the UC Davis Medical Center until June 2007. If additional funding is awarded, the team will build several next-generation systems with 10 separate (parallel) PCR reaction chambers to enable asynchronous sample processing. Asynchronous processing will, in turn, allow sample throughput that is better aligned with the requirements of busy hospitals, especially during the influenza season or in the event of a pandemic influenza.

The flu season arrives every year without fail, bringing fever, coughing, and runny noses. Soon perhaps, FluID$_x$ will be a workhorse application, helping doctors decide how best to care for their patients.

—Katie Walter

Key Words: Autonomous Pathogen Detection System, FluID$_x$, influenza, KPATH, multiplex assays, point-of-care diagnosis, polymerase chain reaction (PCR), respiratory disease.

For further information contact Mary McBride (925) 423-2299 (mcbride2@llnl.gov).